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1 **Crohn's disease siblings exhibit a biologically relevant dysbiosis in mucosal**
2 **microbial metacommunities**

3

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34 **Data deposition**

35 The sequence data reported in this paper have been deposited in the NCBI Short Read Archive database
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37

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39

40 **Author contributions**

41 CRH: study concept and design; obtained funding; recruitment of participants and acquisition of data;
42 analysis and interpretation of data; statistical analysis; drafting of the manuscript; critical revision of the
43 manuscript for important intellectual content. CvdG: Analysis and interpretation of data; statistical analysis;
44 drafting of the manuscript; critical revision of the manuscript for important intellectual content. GBR: DNA
45 extraction; analysis and interpretation of data; statistical analysis; critical revision of the manuscript for
46 important intellectual content. LC: analysis and interpretation of data; statistical analysis. SM: assistance
47 with recruitment of participants; critical revision of the manuscript for important intellectual content. AJS,
48 JOL and KW: study concept and design; obtained funding; analysis and interpretation of data; critical
49 revision of the manuscript for important intellectual content; study supervision.

50

51 **ABSTRACT**

52 Objective

53 Siblings of patients with Crohn's disease (CD) have elevated risk of developing CD and display aspects of
54 disease phenotype, including faecal dysbiosis. Whether the mucosal microbiota is disrupted in these at-risk
55 individuals is unknown. Objective: To determine the existence of mucosal dysbiosis in siblings of CD
56 patients using 454 pyrosequencing and to comprehensively characterise, and determine the influence of
57 genotypic and phenotypic factors, on that dysbiosis.

58 Design

59 Rectal biopsy DNA was extracted from 21 patients with quiescent CD, 17 of their healthy siblings and 19
60 unrelated healthy controls. Mucosal microbiota was analysed by 16S rRNA gene pyrosequencing and were
61 classified into core and rare species. Genotypic risk was determined using Illumina Immuno BeadChip,
62 faecal calprotectin by ELISA and blood T-cell phenotype by flow cytometry.

63 Results

64 Core microbiota of both CD patients and healthy siblings were significantly less diverse than controls.
65 Metacommunity profiling (Bray-Curtis (S_{BC}) index) showed the sibling core microbial composition to be
66 more similar to CD ($S_{BC}=0.70$) than to HC, whereas the sibling rare microbiota was more similar to HC
67 ($S_{BC}=0.42$). *Faecalibacterium prausnitzii* contributed most to core metacommunity dissimilarity both
68 between siblings and controls, and between patients and controls. Phenotype/genotype markers of CD-risk
69 significantly influenced microbiota variation between and within groups, of which genotype had the largest
70 effect.

71 Conclusion

72 Individuals with elevated CD-risk display mucosal dysbiosis characterised by reduced diversity of core
73 microbiota and lower abundance of *F. prausnitzii*. This dysbiosis in healthy people at-risk of CD implicates
74 microbiological processes in CD pathogenesis.

75 **SUMMARY BOX**

76 What is already known about this subject:

- 77 • Patients with CD have mucosal dysbiosis, including reduced abundance of *Faecalibacterium*
78 *prausnitzii*
- 79 • Low mucosal *Faecalibacterium prausnitzii* predicts relapse after surgery in CD patients
- 80 • Healthy siblings of CD patients have increased risk of developing CD and have altered abundance of
81 key species in the gut lumen

82 What are the new findings:

- 83 • There is a distinct dysbiosis in the mucosal microbiota of healthy siblings of CD patients
- 84 • The sibling dysbiosis comprises a fundamental distortion of microbial community composition,
85 most notably reduced diversity of core microbiota and low abundance of mucosal *Faecalibacterium*
86 *prausnitzii*
- 87 • Mucosal microbiota disruption is not merely a consequence of the inflammation in CD but is
88 present at healthy individuals at risk of CD

89

90 How might it impact on clinical practice in the foreseeable future?

- 91 • Identification of this at risk dysbiosis signals pathways in CD pathogenesis and raises the possibility
92 of CD risk identification and CD risk intervention

93 INTRODUCTION

94 Disruption of gut microbiota (dysbiosis) is an established feature of inflammatory bowel disease (IBD). The
95 dysbiosis in Crohn's disease (CD) has been well described and includes reduced microbial diversity, reduced
96 abundance of Firmicutes particularly *Faecalibacterium prausnitzii*, reduced abundance of Bifidobacteria,
97 increased γ -proteobacteria and disturbances in Bacteroides populations.[1] The involvement of several CD
98 susceptibility genes in the recognition and handling of bacteria (e.g., NOD2, ATG16L1, IRGM) reinforces the
99 position of the gut microbiota at the centre of IBD pathogenesis. [2, 3, 4]

100 Whether the CD dysbiosis is involved with pathogenesis is uncertain. The dependence on the presence of
101 gut microbiota for the development of inflammation in animal models[5] as well as CD patients,[6] and the
102 association between reduced mucosal *F. prausnitzii* and post-operative relapse[7] implies a pathogenic
103 role. Conversely, the lack of therapeutic benefit of manipulating the microbiota,[8,9] suggests that
104 dysbiosis in CD may not drive inflammation, but rather is consequent to established disease, reflecting for
105 example, the differential survival of various species in an inflamed environment. Moreover, attempts to
106 identify aspects of the CD dysbiosis that were present at disease initiation, which therefore potentially have
107 a role in pathogenesis may be obfuscated by both the mature disease phenotype of the patients studied
108 and the effect of the medical, surgical and patient-initiated attempts to treat and control symptoms.

109 Siblings of CD patients have a relative risk (RR) of developing CD of up to 35 times that of the general
110 population.[10] This risk is partly genetic, but is also driven by non-genetic factors many of which they
111 share with their CD-affected sibling.[10,11] Several of these non-genetic risk factors, such as mode of
112 delivery, breast feeding, maternal inoculum, home environment and weaning diet,[12] potentially impact
113 gut microbial acquisition and development. It follows that any aspect of the CD dysbiosis which is also
114 present in a healthy sibling cannot be disrupted as a consequence of disease, and rather may be implicated
115 in processes driving CD pathogenesis.[12]

116 Attempts have been made to determine whether aspects of the CD phenotype are present in patients'
117 unaffected relatives. These have assessed dysbiosis[13] and other features of the CD phenotype such as
118 raised faecal calprotectin (FC), increased intestinal permeability (IP) and the presence of anti-microbial
119 antibodies.[12] Using PCR probes selected to detect dominant species that comprise the dysbiosis in CD,
120 we have previously indicated that a faecal dysbiosis exists in healthy siblings of CD patients characterised by
121 reduced faecal Firmicutes including *F. prausnitzii*. [14] Moreover, we previously demonstrated in siblings
122 that a combination of luminal dysbiosis, raised FC, reduced abundance of circulating naïve T-cells,
123 disturbances in their expression of gut-homing $\beta 7$ integrin and at-risk genotype could be combined to
124 create a multidimensional risk phenotype, which significantly distinguished healthy siblings of CD patients
125 from healthy, unrelated controls.[14]

126 It has been speculated that mucosal microbiota are of greater significance in CD pathogenesis than luminal
127 microbiota given their closer spatial relationship to the gut immune system. Yet, studies comparing
128 mucosal microbiota in CD patients, their families and healthy controls are rare due to the invasiveness of
129 procedures required to obtain mucosal samples from otherwise healthy individuals. However, the potential
130 rewards of obtaining such samples have been amplified by recent advances in the analysis of large, diverse
131 and complex microbial communities. Pyrosequencing technology and meta-community profiling enables
132 sampling depth permitting detection not only of dominant microbial community members but also low-
133 abundance (rare) taxa.[15, 16] The capacity to characterise core and rare microbial communities separately
134 may reveal microbial features associated with disease not otherwise readily apparent. Furthermore, 16S
135 rRNA gene pyrosequencing and other next-generation technologies have demonstrated that microbial
136 diversity can be orders of magnitude higher than previously appreciated.[16] Measuring diversity may be
137 significant as healthy gut microbiota high diversity compared with microbial populations in other human
138 body habitats.[18] Moreover, gut microbial diversity is consistently described as reduced both in CD,¹ and
139 other human diseases including obesity,[18, 19] colorectal cancer,[20] eczema,[21] and in addition has
140 been linked with smoking.[22]

141 Therefore, we sought to use 454 pyrosequencing and metacommunity analysis to comprehensively
142 characterise the structure and composition of the mucosal microbial community in an at-risk group of CD
143 siblings compared with CD patients and healthy controls.

144 **MATERIALS AND METHODS**

145 Patients with inactive CD (Crohn's Disease Activity Index (CDAI) <150 and C-reactive protein (CRP) ≤5mg/L,
146 and their healthy siblings (both 16-35 years) were recruited from clinics at Barts Health NHS Trust and
147 University College Hospitals NHS Foundation Trust (London, UK). Patients required a confirmed diagnosis of
148 CD for >3months. All healthy siblings who volunteered and did not meet exclusion criteria (detailed in
149 supplementary Table S1) were included, to limit bias in the selection of siblings with specific characteristics.
150 Healthy controls were recruited by email sent to staff and students at King's College London (London, UK),
151 during the same period. Participants were informed that involvement in the study did not constitute
152 screening for disease and that detection of clinical disease in any sibling or control would lead to exclusion
153 from the study.

154 Only participants consenting to rectoscopy and providing analysable biopsies were included. All participants
155 provided written, informed consent. Ethical approval was provided by Bromley Local Research Ethics
156 Committee (reference 07/H0805/46).

157 At screening, demographics, medical and drug exposure history, physical examination, CRP, inclusion and
158 exclusion criteria were assessed. Instructions regarding avoidance of prebiotics/probiotics for 4 weeks (to
159 prevent impact on microbiota), non-steroidal anti-inflammatory drugs for 1 week and alcohol for 24h
160 before the study (to prevent impact on IP) were provided. Blood samples were taken for routine
161 haematology/biochemistry, T-cell analysis and genotyping. Participants completed a 5h urine collection for
162 measurement of IP and underwent flexible rectoscopy without bowel cleansing. Biopsies from non-
163 inflamed rectum were snap frozen, and stored at -80°C before processing for histological and
164 microbiological analyses. Stool was obtained and stored at -20°C before processing for FC quantification.

165 **Faecal calprotectin**

166 FC extraction and ELISA analysis (Calpro AS, Lysaker, Norway) were carried out according to manufacturer's
167 instructions using duplicate appropriately diluted samples. FC concentration ($\mu\text{g/g}$) was determined relative
168 to standard curves.

169 **Peripheral blood T-cell flow cytometry**

170 Whole blood, collected in lithium-heparin Vacutainer tubes (BD Bioscience), was stored at room
171 temperature for ≤ 4 h before labelling with fluorescently conjugated monoclonal antibodies to detect CD3 T-
172 cells, naïve (CD45RA^+) and memory (CD45RA^-) subsets of CD4 and CD8 T-cells. Integrin $\alpha 4\beta 7$ expression was
173 assessed by labelling with anti- $\beta 7$ (see supplementary methods for antibodies used). Data were acquired
174 using a LSRII 4-colour flow cytometer (BD Bioscience) and collected using fluorescence-activated cell sorting
175 Diva software V.4.1.2 (BD Bioscience) using Flow-Count fluorospheres (Beckman Coulter) for absolute
176 quantitation. Colour compensation was performed offline using Winlist V.6.0 (Verity Software House).

177 **Genotyping**

178 Human DNA was extracted from whole blood using the phenol chloroform-isoamyl alcohol method.
179 Genotyping was performed using the Illumina Infinium ImmunoChip.[2, 23] To increase detection of NOD2
180 mutations and capture the enhanced risk of NOD2 compound heterozygosity, three NOD2 mutations
181 (rs2066845/G908R, rs2066844/R702W and rs5743293/3020insC) were individually assessed. Cumulative
182 genotype relative risk (GRR) for each participant was therefore calculated across 72 CD-risk loci. A
183 population distribution model of CD-risk was generated using the REGENT R program[24] and previously
184 published odds ratios.[2] Participants were categorised into reduced, average, elevated or high genotype
185 risk with reference to this model.[25]

186 **Intestinal permeability**

187 IP was measured using lactulose-rhamnose tests as previously described.[14]

188 **Gut mucosal microbiota**

189 Biopsy DNA extraction was carried out using a phenol/chloroform based method, as described
190 previously.[26] A detailed extraction protocol is provided in the supplementary methods. DNA extracts
191 were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). Negative controls
192 (sterile water), were included in the DNA extraction and PCR amplification steps.

193 Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described previously using
194 Gray28F 5'-TTTGATCCTGGCTCAG-3' and Gray519r 5'-GTNTTACNGCGGCKGCTG-3'.[27] Detailed protocols
195 for 16S rRNA gene sequencing and sequence data processing are provided in the supplementary methods.

196 To assign bacterial identities to 16S rRNA gene sequences, sequence data were de-noised, assembled into
197 OTU clusters at 97% identity, and queried using a distributed .NET algorithm that utilises Blastn+
198 (KrakenBLAST, www.krakenblast.com) against a database of high quality 16S rRNA gene bacterial
199 sequences. Using a .NET and C# analysis pipeline the resulting BLASTn+ outputs were compiled, data
200 reduction analysis performed, and sequence identity classification carried out, as described previously.[28]

201 **Statistical analyses**

202 Bacterial species within each metacommunity were partitioned into common and rare groups using a
203 modification of a previously described method.[15] Three complementary measurements of diversity were
204 used to compare microbial diversity between samples, as previously described: species richness (S^* , the
205 total number of species), Shannon-Wiener (H' , a metric accounting for both number and relative
206 abundance of species), and Simpson's (1-D, a measure of the probability that two species randomly
207 selected from a sample will differ).[15, 26] To avoid potential bias due to varying sequences per sample, all
208 measures were calculated using randomised re-sampling to a uniform number of sequence reads per
209 sample.[26] Mean diversity measures were calculated from the re-sampling of the reads from each
210 specimen to the lowest number of sequence reads among all specimens for 1000 iterations. Diversity
211 analysis was performed in R.[29] Two sample t-tests, regression analysis, coefficients of determination (r^2),

212 residuals and significance (P) were calculated using Minitab software (version 16, Minitab, University Park,
213 PA, USA). Canonical correspondence analysis (CCA), analysis of similarity (ANOSIM), similarity of
214 percentages (SIMPER) analysis were performed using the PAST (Palaeontological Statistics, version 3.01)
215 program available from the University of Oslo website link (<http://folk.uio.no/ohammer/past>) run by
216 Øyvind Hammer. The Bray-Curtis quantitative index of similarity was used as the underpinning community
217 similarity measure for CCA, ANOSIM, and SIMPER tests.

218 **RESULTS**

219 Demographic and disease characteristics of the 21 patients with quiescent CD, 17 of their healthy siblings,
220 and 19 unrelated healthy controls that were included are summarised in Table 1. At the time of the study
221 only one patient was cohabiting with one of the included siblings. GRR, FC, faecal Firmicute abundance and
222 circulating T-cell characteristics were all significantly different in both CD patients and healthy siblings
223 compared with healthy controls as previously published,[14] and as summarised in Table 1.

224

225 **Table 1 Summary of demographic variables in patients, siblings and controls as well as clinical**
226 **characteristics in patients. The features of the at-risk phenotype that have previously been delineated in**
227 **this cohort are also displayed.**

		Patients (n=21)	Siblings (n=17)	Controls (n=19)	P-value
Mean age years, (SD)		27.7 (6.6)	25.5 (4.5)	27.7 (5.8)	0.783*
Males, n (%)		13 (62)	11 (65)	9 (47)	0.515†
Body Mass Index, kg/m ² (SD)		24.5 (5.0)	24.5 (3.6)	23.9 (3.4)	0.870*
Ethnicity n (%)	White British	17 (81)	15 (88)	17 (90)	0.469†
	Asian/Asian British	3 (14)	1 (6)	0 (0)	
	Black British or mixed black/white	1 (5)	1 (6)	2 (11)	
Smoking n (%)	Never	14 (67)	10 (59)	12 (63)	0.830†
	Current	4 (19)	5 (29)	3 (16)	
	Previous	3 (14)	2 (12)	4 (21)	
Age at diagnosis, n (%)	Below 16 years	7 (33)			
	16-40 years	14 (67)			
Disease location, n (%)	Ileal	7 (33)			
	Colonic	5 (24)			
	Ileocolonic	9 (43)			
Concomitant upper GI disease, n (%)		1 (5)			
Disease behaviour, n (%)	Non-stricturing, non-penetrating	11 (52)			
	Stricturing	5 (24)			
	Penetrating	5 (24)			
Perianal disease, n (%)		4 (19)			
Current 5-ASA n (%)		11 (52)			
Current immuno-suppressant, n (%)	Azathioprine	7 (33)			
	Mercaptopurine	2 (10)			
	Methotrexate	1 (5)			
Ileocaecal resection / right hemicolectomy, n (%)		9 (43)			
Isolated small bowel resection, n (%)		1 (5)			
Genotype relative risk, n (%)	High	3 (14)	1 (6)	0 (0)	0.175†
	Elevated	2 (10)	3 (18)	0 (0)	
	Average	10 (48)	8 (47)	8 (42)	

Reduced	6 (29)	5 (29)	11 (58)	
Fecal calprotectin, m/g (IQR)	281 (144-855)	30 (13-83)	13 (7-33)	<0.001‡
Faecal <i>F. prausnitzii</i> , % (IQR)	0.1 (0.0-2.9)	3.7 (0.4-7.1)	5.2 (2.3-7.2)	0.001‡
T-cells with memory phenotype, % (IQR)	73 (63-82)	74 (67-83)	65 (54-70)	0.011‡
Naïve CD4 ⁺ T-cells, cells/ ml (IQR)	194,132 (71,053-341,156)	198,220 (128,550-296,351)	380,256 (279,118-564,861)	<0.001‡
Naïve CD4 ⁺ T-cells expressing β 7 integrin, % (IQR)	76 (63-85)	74 (61-83)	52 (32-71)	0.003‡
Intestinal permeability: urinary lactulose-rhamnose ratio, (IQR)§	0.061 (0.033-0.111)	0.034 (0.024-0.056)	0.038 (0.025-0.050)	0.081‡

* One-way ANOVA

† Chi-squared test

‡ Kruskal-Wallis test

§ Data from 20 patients, 17 siblings and 16 controls contributed to the intestinal permeability analysis

|| Cumulative genotype relative risk (GRR) for each participant was calculated across 72 CD-risk loci (detected using the Illumina Infinium Immunochip). Participants were categorised into reduced, average, elevated or high genotype risk with reference to a population distribution model of CD-risk, previously described.[14]

236 A total of 180,696 bacterial sequence reads (mean per sample $3235 \pm \text{SD } 205$), identifying 160 genera and
237 351 distinct operational taxonomic units (OTUs) classified to species level (Table S2), were generated from
238 all samples combined. The numbers of bacterial sequence reads per sample were similar among the three
239 cohorts (mean \pm SD): CD, 3296 ± 258 (n =21); siblings, 3190 ± 423 (n =17); and healthy, 3210 ± 393 (n =19).

240 **Species abundance was directly correlated with distribution**

241 We have previously established that the categorisation of human microbiota into core and rare species
242 revealed important aspects of metacommunity species-abundance distributions that would be neglected
243 without such a distinction.[15] A coherent metacommunity could be expected to exhibit a direct
244 relationship between prevalence and abundance of individual species within the constituent communities.
245 Consistent with this prediction, the abundance of species in each study group significantly correlated with
246 the number of individual sample communities those species occupied (CD ($R^2 = 0.62$, $F_{1,227} = 366.9$, $P <$
247 0.0001); siblings ($R^2 = 0.71$, $F_{1,259} = 590.1$, $P < 0.0001$); and healthy controls ($R^2 = 0.68$, $F_{1, 258} = 552.6$, $P <$
248 0.0001)), (Fig. 1).

249 **In CD patients a lower proportion of the mucosal microbiota were core species**

250 Individual species in each cohort metacommunity were then classified as core or rare based on their falling
251 within or outside the upper quartile of subject occupancy, respectively (Fig. 1). Of the 229 species that
252 comprised the CD metacommunity, only 7 were core and 222 were rare species. The healthy siblings
253 metacommunity (261 species) comprised 18 core and 243 rare species, and the healthy controls
254 metacommunity (260 species) comprised 25 and 235 species, respectively. In addition, the core species
255 within each cohort metacommunity accounted for $44.7\% \pm 4.8\%$ (CD), $67.6\% \pm 5.5\%$ (healthy siblings) and
256 $67.4\% \pm 4.6$ (healthy controls) of the mean (\pm SD) relative abundance. The mean relative abundances in the
257 CD core microbiota were significantly lower than the healthy siblings and healthy controls ($P < 0.0001$ in
258 both cases), but were not different between the siblings and healthy controls ($P = 0.907$).

259 **Microbial diversity was lower in both siblings and patients compared with controls**

260 The mean microbial diversity of subject communities for each cohort was compared using three indices of
261 diversity (Fig. 2). Diversity was compared between the three cohorts for the whole microbiota, as well as
262 core and rare species groups (Fig. 2). These analyses revealed the siblings' whole and core microbiota to be
263 significantly more diverse than the CD cohort, but the sibling core microbiota was significantly less diverse
264 than the healthy core microbiota. No significant difference in diversity was observed between the whole
265 microbiota between the siblings and healthy cohorts, emphasising the advantage of analysing core and rare
266 populations separately. In addition, the CD rare microbiota was significantly less diverse than the other two
267 rare species cohorts, which in turn were not significantly different from each other. All of these
268 observations were underpinned by all three measures of diversity in each instance (Fig. 2).

269 Interestingly, within the CD population, diversity of the whole microbiota was lower in the nine patients
270 with an ileocaecal resection / right hemicolectomy compared with the 11 patients without these operations
271 (as shown by Richness $P < 0.0001$; Shannon-Wiener $P = 0.046$; but not Simpson's $P = 0.768$). This was largely
272 driven by lower diversity of rare taxa (as shown by Richness $P < 0.0001$; Shannon-Wiener $P = 0.019$; but not
273 Simpson's $P = 0.159$) rather than core taxa (Richness $P = 0.523$; Simpson's $P = 0.612$; Shannon-Wiener
274 $P = 0.824$).

275 **Significant divergence in whole and core microbial composition between CD patients and healthy**
276 **controls, but not between CD patients and healthy siblings**

277 The distribution of the microbiota within the three cohorts was determined by direct ordination using Bray-
278 Curtis similarity measures. Using Analysis of Similarities (ANOSIM) tests, the CD and healthy whole and core
279 microbiota were significantly divergent from each other. However, the whole and core microbiota of
280 siblings were not significantly divergent from either that of the CD or healthy controls (Fig. 3). In all
281 instances rare microbiota were significantly divergent between cohorts, including between siblings and
282 healthy controls.

283 **Lower *Faecalibacterium prausnitzii* made the greatest contribution to the dissimilarity in microbiota**
284 **between both healthy siblings and healthy controls and between CD and healthy controls**

285 Given the involvement of core species in differences of relative abundance, diversity and microbiota
286 composition, the contribution of individual taxa to the dissimilarity between core microbiota was assessed
287 by Similarities of Percentages (SIMPER) analyses (Table 2). Both *F. prausnitzii* and *Escherichia fergusonii*
288 contributed the most to the dissimilarity between all cohorts. As a proportion of core species *F. prausnitzii*
289 had a higher relative abundance in the healthy controls (30.9%) than both the CD (22.4%) and siblings
290 (24.2%). Conversely, *E. fergusonii* was more abundant in the CD cohort (21.4%) than in siblings (9.7%) and
291 healthy controls (4.1%).

292

293 **Table 2 Similarity of Percentages (SIMPER) analysis of microbial community dissimilarity (Bray-Curtis)**
294 **between core species groups for (A) CD and siblings, (B) healthy and siblings, and (C) CD and healthy**
295 **cohorts.** Given is mean % abundance of sequences for core species only across the samples each was
296 observed to occupy and the average dissimilarity between samples (overall mean (A) =73.4% and (B)
297 =55.0%, (C) =73.0%). Percentage contribution is the mean contribution divided by mean dissimilarity across
298 samples. The list of species is not exhaustive so cumulative % value does not sum to 100%. Species level
299 identities of detected taxa are reported here. However, given the length of the ribosomal sequences
300 analysed, these identities should be considered putative.

A	Crohn's	Siblings		
Name	mean abundance	mean abundance	% Contribution	Cumulative %
<i>Faecalibacterium prausnitzii</i>	22.4	24.2	20.7	20.7
<i>Escherichia fergusonii</i>	21.4	9.7	15.9	36.6
<i>Shigella flexneri</i>	13.6	7.2	10.7	47.3
<i>Ruminococcus gnavus</i>	13.1	5.2	8.9	56.2
<i>Bacteroides vulgatus</i>	13.2	7.6	7.8	64.0
<i>Eubacterium rectale</i>	9.8	6.4	6.6	70.6
<i>Oscillospira guilliermondii</i>	0	8.0	5.9	76.5
<i>Escherichia coli</i>	6.5	0	4.5	81.0
<i>Sutterella wadsworthensis</i>	0	6.0	4.5	85.5
<i>Bacteroides dorei</i>	0	5.7	4.2	89.6
<i>Roseburia faecis</i>	0	4.0	2.9	92.6
B	Healthy	Siblings		
Name	mean abundance	mean abundance	% Contribution	Cumulative %
<i>Faecalibacterium prausnitzii</i>	30.9	24.2	18.9	18.9
<i>Escherichia fergusonii</i>	4.1	9.7	10.6	29.5
<i>Sutterella wadsworthensis</i>	8.7	6.0	9.4	38.9
<i>Shigella flexneri</i>	3.6	7.2	8.4	47.3
<i>Bacteroides vulgatus</i>	8.0	7.6	8.4	55.7
<i>Eubacterium rectale</i>	9.9	6.4	7.0	62.8
<i>Oscillospira guilliermondii</i>	8.5	8.0	7.0	69.8
<i>Bacteroides dorei</i>	0	5.7	5.4	75.2
<i>Ruminococcus gnavus</i>	4.1	5.2	4.1	79.3
<i>Bacteroides uniformis</i>	2.9	2.0	3.1	82.4
<i>Roseburia faecis</i>	2.4	4.0	3.0	85.4
<i>Coprococcus eutactus</i>	2.3	0	2.2	87.7
<i>Shigella dysenteriae</i>	2.1	0	2.1	89.8
<i>Blautia producta</i>	2.0	1.8	1.8	91.6
C	Crohn's	Healthy		
Name	mean abundance	mean abundance	% Contribution	Cumulative %
<i>Faecalibacterium prausnitzii</i>	22.4	30.9	22.4	22.4
<i>Escherichia fergusonii</i>	21.4	4.1	14.4	36.7
<i>Shigella flexneri</i>	13.6	3.6	9.3	46.0
<i>Ruminococcus gnavus</i>	13.1	4.1	8.6	54.6
<i>Bacteroides vulgatus</i>	13.2	8.0	8.0	62.7
<i>Eubacterium rectale</i>	9.8	9.9	7.4	70.1
<i>Sutterella wadsworthensis</i>	0	8.7	6.4	76.5
<i>Oscillospira guilliermondii</i>	0	8.5	6.2	82.8
<i>Escherichia coli</i>	6.5	0	4.4	87.2
<i>Bacteroides uniformis</i>	0	2.9	2.1	89.3
<i>Roseburia faecis</i>	0	2.4	1.8	91.1

301

302 **Genotype and phenotypic features associated with CD and CD-risk significantly explained microbiota**
303 **variation**

304 Canonical correspondence analysis (CCA) was used to relate the variability in the distribution of microbiota
305 between cohorts to clinical and demographic variables (Table 3 and Fig. 4). Variables that significantly
306 explained variation in mucosal microbiota were determined with forward selection (999 Monte Carlo
307 permutations; $P < 0.05$) and used in CCA. Based on the direct ordination approach, the microbiota between
308 cohorts was significantly influenced by factors listed in Table 3. The same analytical approach was used to
309 assess the extent to which variance in the microbiota distribution within cohorts could be accounted for by
310 variation in measures of clinical and demographic factors, (Table 3). GRR was the most significant factor in
311 explaining variance between the three cohorts, but also within each cohort. FC was also significant in
312 explaining variance between cohorts, particularly in the core microbiota. However, in the within-group
313 analyses FC was significant in explaining microbial variance in patients and siblings but not in controls.
314 Blood T-cell factors explained a higher proportion of variance in siblings and controls than in patients.
315 Conversely, age significantly associated with variance in controls but not in patients or siblings.

316 **Table 3 Canonical correspondence analyses for determination of percent variation in the whole, core, and rare microbiota between and within the three**
317 **subject cohorts by clinical variables significant at the P < 0.05 level.** * Ileal/Colonic involvement in CD patients used as a factor for the corresponding siblings. n/a
318 denotes not applicable for between cohort or within healthy cohort analyses.

Variable	Between Cohorts			Within Crohn's			Within Siblings			Within Healthy		
	Whole	Core	Rare	Whole	Core	Rare	Whole	Core	Rare	Whole	Core	Rare
Age	-	-	-	-	-	-	-	-	-	5.37	6.08	5.08
Blood concentration of naïve CD4 ⁺ T-cells (cells /ml)	-	2.8	-	4.15	3.69	4.54	7.34	8.84	8.30	-	-	-
Calprotectin	1.7	3.4	2.7	4.68	5.36	4.63	4.85	5.70	7.10	-	-	-
Gender	1.8	-	2.4	5.03	-	6.20	6.04	9.12	7.03	6.31	4.57	7.19
Genotype relative risk (GRR)	5.1	4.3	4.7	8.56	6.75	9.11	6.53	12.54	5.64	5.57	6.26	5.34
Ileal/Colonic involvement*	n/a	n/a	n/a	5.50	-	5.87	4.94	6.40	5.81	n/a	n/a	n/a
Proportion of blood T-cells with memory phenotype (%)	2.0	-	2.4	-	-	-	8.71	5.77	9.24	5.19	-	6.87
Proportion of CD4 ⁺ naïve T-cells expressing β7 integrin (%)	-	3.6	-	4.85	5.84	5.19	5.30	-	7.81	5.60	10.08	4.15
Undetermined	89.4	85.9	87.8	67.2	78.4	64.5	56.3	51.6	49.1	72.0	73.0	71.4

319 * Ileal/Colonic involvement in Crohn's patients used as a factor for the corresponding Sibling subjects. n/a denotes not applicable for between cohort or within
320 Healthy cohort analyses.

321 DISCUSSION

322 This is the first study to detail the mucosal microbiota of clinically and genetically well-characterised
323 healthy siblings of CD patients, and to compare them with both their CD-affected siblings and healthy
324 controls. Moreover, this study is unique in uncovering interactions of mucosal microbiota with genotype
325 and features of the CD-risk phenotype. This manuscript is a significant advance on the preliminary account
326 of the multidimensional risk phenotype previously described, which centred on qPCR sampling of faecal
327 microbiota.[14] The current study not only focuses on the mucosal microbiota but also employs next-
328 generation sequencing and advanced statistical analysis to reveal the complexity of the metacommunities
329 in healthy siblings of CD patients. The core mucosal microbiota in siblings was characterised by lower
330 diversity compared with controls, and lower abundance of *F. prausnitzii* made the greatest contribution to
331 the dissimilarity between these two groups. Genetic CD-risk explained the highest proportion of microbial
332 variance both between all three groups, and within the patient and sibling groups. These findings are
333 unlikely to be confounded by cohabitation as only one patient cohabited with one sibling.

334 Although related healthy individuals are known to harbour similar gut microbiota,[19] the similarity in the
335 microbiota between CD patients and their healthy siblings is of considerable pathogenic relevance. Previous
336 studies have shown that when one sibling has CD, familial microbial similarity is disrupted, even in disease-
337 discordant monozygotic twins.[30] Thus, microbial features which are similar between affected and
338 unaffected siblings, but which are not present in low CD-risk healthy individuals, may be part of the CD-risk
339 phenotype and therefore pertinent to CD pathogenesis. In order to discern these features associated with
340 familial risk, comparison with healthy, unrelated individuals is essential.

341 The validity of the data presented is supported by the correlation between species-abundance and
342 distribution, which is consonant with a coherent metacommunity structure and is similar to distributions
343 described in other ecological communities.[15] This feature of community structure facilitated delineation
344 of core species which are abundant and persistent, and allowed resolution of features of the mucosal
345 microbiota without obfuscation from rare microbiota which may be highly variable, transient and scarce. A

346 significantly higher proportion of the microbiota in CD patients belonged to the rare group compared with
347 healthy siblings and healthy controls. As described below this is at least in part attributable to loss of
348 principal members of the core group, most notably Firmicutes.

349 Reduced microbial diversity is an almost universally reported feature of mucosal CD dysbiosis.[1] The
350 current study reveals that core microbiota diversity is also lost in siblings of CD patients, indicating that this
351 may be a fundamental step in CD pathogenesis. Reduced diversity may be an indicator of the health of
352 human microbial communities, as it is reduced in a variety of disorders.[18-21] Lower diversity may be
353 associated with incomplete occupation of ecological niches resulting in reduced resistance to pathogen
354 colonisation; additionally a more restricted gut metagenome contains a lower array of genes which may
355 result in the loss of key functions.

356 Lower diversity indicates altered mucosal microbial composition, and microbial composition in CD patients
357 and healthy controls were significantly distinct from one another. In contrast, the composition of the whole
358 and core microbiota in healthy siblings was not significantly different from either CD patients or healthy
359 controls, indicating that from a microbial metacommunity perspective, siblings lie somewhere between
360 patients and controls. The greater variability in the composition of the microbiota in at-risk siblings
361 (illustrated by larger 95% concentration ellipse in Figure 3 (panel B)) probably reflects the range of CD-risk
362 contained within this group, with siblings with higher CD-risk lying closer to or within the CD region. In
363 addition, diversity was lower in core and rare microbiota in patients with ileocaecal resection/ right
364 hemicolectomy, potentially explained by differences in disease phenotype, or the absence of the ileocaecal
365 valve that would otherwise constitute a barrier between small and large intestinal microbiota.

366 Consonant with previous work highlighting the importance of *F. prausnitzii* in CD dysbiosis,[7, 12, 14] *F.*
367 *prausnitzii* made the greatest contribution to the dissimilarity between CD patients and healthy control
368 microbiota. The prominence of *F. prausnitzii* has biological significance as it is the only microbial factor
369 shown to be predictive of the natural history of CD,[7] and response to treatment.[31] Strikingly, *F.*

370 *prausnitzii* was also the biggest contributor to the dissimilarity of the core mucosal microbiota between
371 healthy siblings and healthy controls, establishing that mucosal *F. prausnitzii* not only correlates to the
372 natural history of CD, but is also a key feature of the at-risk phenotype. Taken together these findings
373 strongly support the hypothesis that depletion of *F. prausnitzii* is part of CD pathogenesis rather than
374 consequent to established CD. Several mechanisms exist whereby *F. prausnitzii* and other Firmicutes may
375 contribute to gut health, including the production of short-chain fatty acids (SCFAs),[32, 33] SCFA-
376 independent, NFκB-mediated effects,[7] and via production of longer-chain fatty acids such as conjugated
377 linoleic acid.[34]

378 The pathogenic role of reduced *F. prausnitzii* in CD has been questioned by a study describing increased
379 mucosal *F. prausnitzii* in newly-diagnosed pediatric IBD.[35] However, whether increased abundance of *F.*
380 *prausnitzii* is a distinctive feature of pediatric-onset IBD, with low *F. prausnitzii* being associated with later-
381 onset CD, or whether the abundance of *F. prausnitzii* may bloom in childhood and then critically decline in
382 those at risk of CD, may only be determined by longitudinal studies.

383 Other species contributing to the dissimilarity in the core mucosal microbiota between CD patients and
384 healthy controls were congruent with species previously identified as characterising the CD dysbiosis,
385 including a greater abundance of most Proteobacteria such as *E. fergusonii* and *Escherichia coli*. Similar
386 species contributed to the dissimilarity between siblings and controls. However, the presence of *E. coli* was
387 specific to CD mucosa, and therefore may be a feature of established CD rather than pathogenic. Features
388 of the inflamed gut such as increased activity of nitric oxide synthases[36], or reduction in faecal butyrate
389 producers which will result in a rise in pH, potentially favour the survival of organisms that are inhibited at
390 acidic pH such as *E. coli*.[37]

391 GRR was the factor associated most strongly with the variation in the microbiota in both the between-
392 group analysis, and analysis within each of the three groups. Although the proportion of variation in
393 mucosal microbiota explained by GRR was small, it is nevertheless significant. The combination of loci used

394 to estimate GRR in the current study does not include more recently detected risk loci and can be expected
395 to account for a limited proportion of the genetic risk.[38] Therefore, these data will tend to underestimate
396 the effect of genotype. Furthermore, since other factors known to affect gut microbiota such as diet were
397 not controlled, this signal of the interaction between genotype and the mucosal microbiota is striking.

398 The direction of the vector in figure 3 illustrates that FC contributed to the axis separating patients from the
399 other two groups in the whole, core and rare microbiota, implying that microbial composition in CD is
400 partly associated with the degree of inflammation. This would support the hypothesis that CD-specific
401 elements of the dysbiosis may be consequent to intestinal inflammation, through mechanisms such as the
402 enhanced survival of *E. coli* in an inflamed environment as proposed above.

403 When each group was considered separately, the effect of each factor in different groups could be
404 compared. Several factors were significant in all groups (GRR, gender, proportion of CD4⁺ naïve T-cells
405 expressing β 7 integrin). Other factors were significant in patients and siblings but not controls: FC and
406 blood naïve CD4⁺ T-cell concentration were significant only in patients and siblings, whereas age was
407 significant only in controls. Disease phenotype was significant in explaining microbial variation within the
408 CD group as would be predicted from previous studies.[30] However; we have also demonstrated that for
409 healthy siblings, disease site in their affected relative was significantly associated with the variation in their
410 own microbiota. This would suggest that specific risk phenotypes are associated with different disease
411 phenotypes.

412 Overall these factors accounted for a higher proportion of the variance in the microbial composition in
413 siblings, compared with controls or patients, indicating that this multidimensional risk phenotype is specific,
414 and that in low CD-risk individuals the microbial composition is associated with other factors, such as age.
415 Furthermore, it would appear that in CD the influence of factors associated with the original risk phenotype
416 is obfuscated by established CD and its surgical and medical management.

417 **CONCLUSION**

418 Healthy siblings of CD patients, who themselves have elevated risk of CD, have a dysbiosis of the core
419 mucosal microbiota characterised by reduced diversity and loss of Firmicutes, notably *F. prausnitzii*.
420 Genotype determines a proportion of the at-risk mucosal microbial phenotype. Notwithstanding the
421 limited extent to which known loci account the observed CD-risk,[39] it is also clear that the sibling risk
422 goes beyond genotype and that non-genetic factors within families contribute to the development of an at-
423 risk microbiota. How and why patients and their siblings acquire the microbiota that marks out this risk is
424 not known. However, knowledge of the at-risk microbial phenotype illuminates possible pathways in CD
425 pathogenesis and raises the prospect of intervention to impact human health and influence disease risk.

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430

431 **FIGURE LEGENDS**

432 Fig. 1. The distribution and abundance of bacterial species within microbiota samples within the (a) CD, (b)
433 siblings, and (c) healthy control cohort metacommunities. Given is the number of mucosal samples for
434 which each bacterial taxon was observed to occupy, plotted against the mean abundance across all samples
435 ((a) $n = 21$, $r^2 = 0.62$, $F_{1, 227} = 366.9$, $P < 0.0001$; (b) $n = 17$, $r^2 = 0.71$, $F_{1, 259} = 590.1$, $P < 0.0001$; and (c) n
436 $= 19$, $r^2 = 0.68$, $F_{1, 258} = 552.6$, $P < 0.0001$). Core species were defined as those that fell within the upper
437 quartile (dashed lines), and rare species defined as those that did not.

438

439 Fig. 2. Diversity of whole, core and rare microbiota within the CD (black columns), siblings (grey), and
440 healthy (white)-control cohorts. Given are three indices of diversity; Species richness (S^*), Simpson's index
441 of diversity ($1-D$), and Shannon-Wiener index of diversity (H'). Error bars represent the standard deviation
442 of the mean (CD $n = 21$, siblings $n = 17$, and healthy $n = 19$). Asterisks denote significant differences in
443 comparisons of diversity at the $P < 0.05$ level determined by two sample t -tests.

444

445 Fig. 3. Analysis of similarities (ANOSIM) of whole, common, and rare microbiota between subject cohorts.
446 Given is the ANOSIM test statistic (R) and probability (P) that two compared groups are significantly
447 different at the $P < 0.05$ level (* denotes $P < 0.001$ and ** $P < 0.0001$). ANOSIM R and P values were
448 generated using the Bray-Curtis measure of similarity. R scales from +1 to -1. +1 indicates that all the most
449 similar samples are within the same groups. $R = 0$ occurs if the high and low similarities are perfectly mixed
450 and bear no relationship to the group. A value of -1 indicates that the most similar samples are all outside
451 of the groups.

452

453 Fig. 4. Canonical correspondence biplots for (a) whole, (b) core, and (c) rare microbiota. Red crosses
 454 represent microbiota samples from the CD cohort, yellow filled triangles for the siblings cohort, and green
 455 diamonds for the healthy cohort. In each instance, the 95 % concentration ellipses are given for the CD
 456 (red), siblings (yellow), and healthy (green) cohort microbiota. Biplot lines for clinical variables that
 457 significantly accounted for variation within the microbiota at the $P < 0.05$ level (see Table 3) show the
 458 direction of increase for each variable, and the length of each line indicates the degree of correlation with
 459 the ordination axes. CCA field labels: Calprotectin, Gender, "T-cells" – Proportion of blood T-cell with
 460 memory phenotype (%), "CD4⁺ T-cells" – Blood concentration of naïve CD4⁺ T-cells (cells /ml), "β7 integrin"
 461 – Proportion of CD4 naïve T-cells expressing β7 integrin (%), "GRR" – genotype relative risk, (cumulative
 462 genotype relative risk (GRR) for each participant was calculated across 72 CD-risk loci (detected using the
 463 Illumina Infinium Immunochip), participants were categorised into reduced, average, elevated or high
 464 genotype risk with reference to a population distribution model of CD-risk). Percentage of community
 465 variation explained by each axis is given in parentheses.

466

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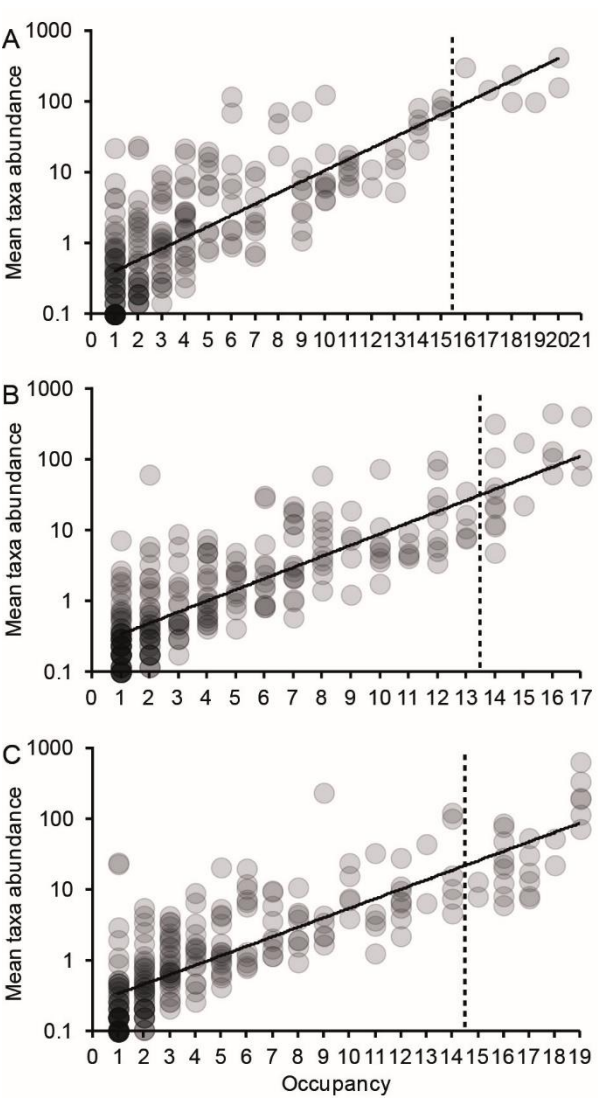
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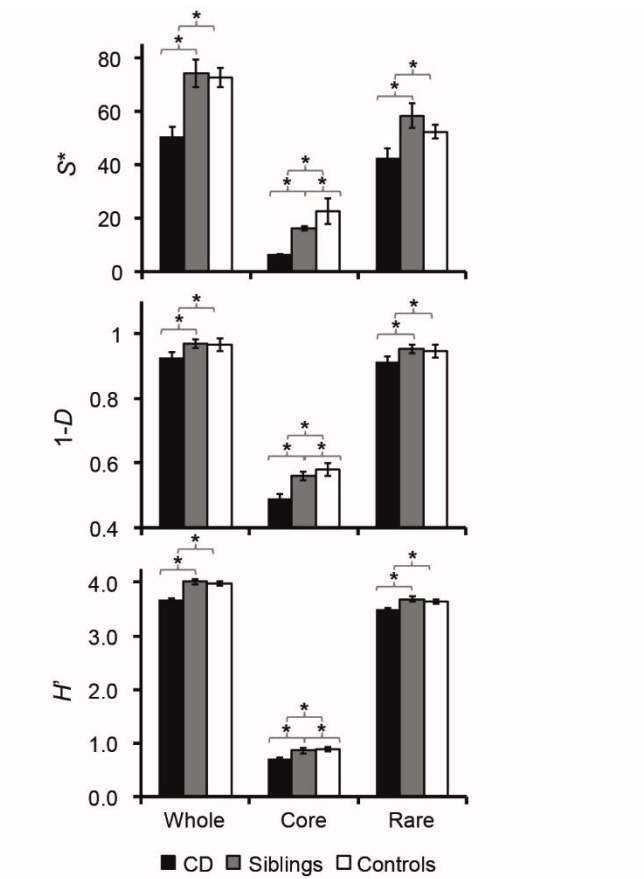
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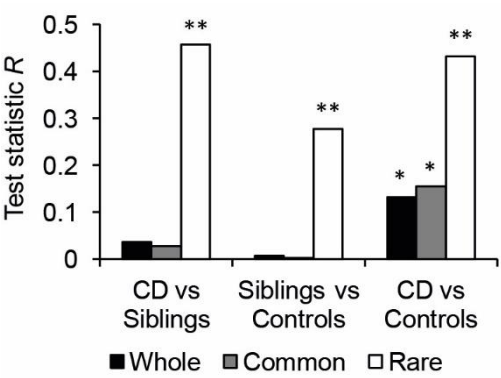
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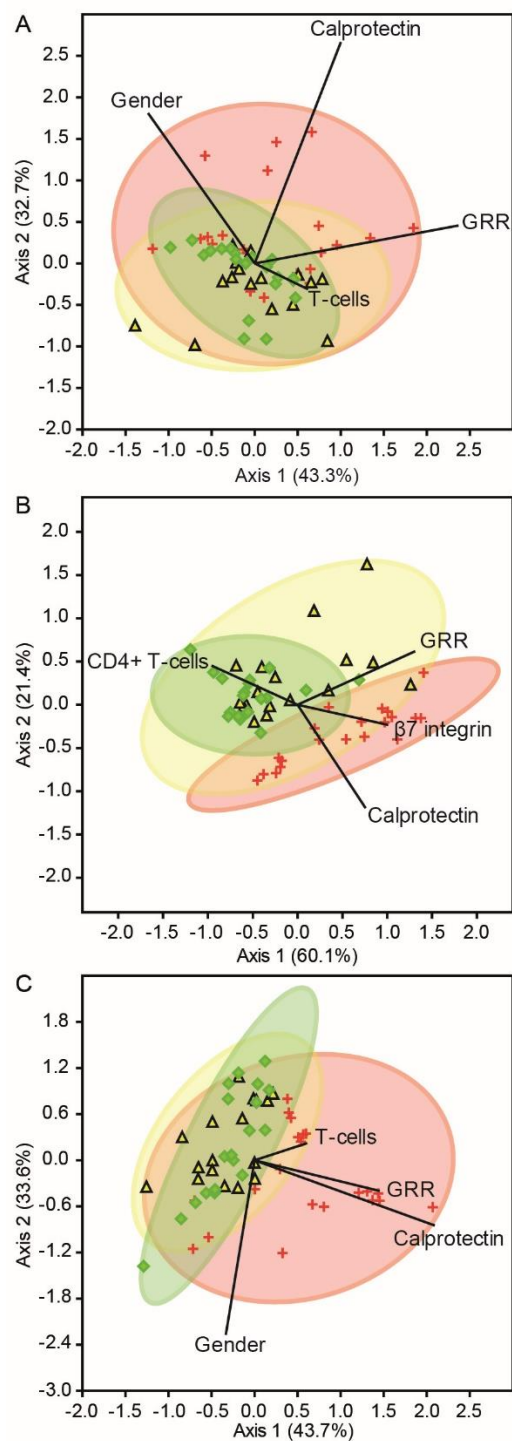
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554 Figure 3



555

556



All participants	Patients	Siblings	Controls
Unable to consent due to mental illness/ dementia/ learning disability	Evidence of active CD as defined by a CDAI of greater than 150	Previous diagnosis of IBD	Previous diagnosis of IBD
Current infection with an enteric pathogen	Purely perianal CD	Symptoms fulfilling Rome III criteria for IBS	Symptoms fulfilling Rome III criteria for IBS
Use of antibiotics within the last month	Change in dose of oral steroids within the last 4 weeks		A first or second degree relative with IBD
Consumption of any probiotic or prebiotic within the last month	Dose of steroids exceeding 10mg prednisolone per day or equivalent		
Pregnancy or lactation	Change in dose of oral 5-ASA products within the last 4 weeks		
Participant requiring hospitalization	Commencement of azathioprine or methotrexate within the last 4 months, or change in dose of these drugs within the last 4 weeks		
Significant hepatic, renal, endocrine, respiratory, neurological or cardiovascular disease as determined by the principal investigator	Infusion of biological therapies (e.g. infliximab) within the last 3 months*		
A history of cancer with a disease free state of less than two years	Use of rectal 5-ASA or steroids within the last 2 weeks		
CRP greater than 5mg/L at screening, as measured by the local laboratory	Use of NSAIDs within the last 2 weeks		
	Imminent need for surgery		
	Short bowel syndrome		
	Previous proctocolectomy		

561 * No patient had been previously exposed to biological therapies

563 **Supplementary table S2** Bacterial species identified from biopsy samples collected from subject
564 within the Crohn's ($n = 21$), Siblings ($n = 17$), and Healthy ($n = 19$) cohorts. Species-level identities
565 of detected taxa are reported here. However, given the length of the ribosomal sequences
566 analysed, these identities should be considered putative. C and R denote core or rare species
567 group membership within each cohort (highlighted in green and yellow, respectively).
568

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
Actinobacteria	Actinobacteria	Actinomycetaceae	<i>Actinomyces naeslundii</i>			R
			<i>Varibaculum cambriense</i>		R	R
		Corynebacteriaceae	<i>Corynebacterium afermentans</i>		R	
			<i>Corynebacterium amycolatum</i>	R	R	R
			<i>Corynebacterium aurimucosum</i>		R	
			<i>Corynebacterium durum</i>	R		
			<i>Corynebacterium glucuronolyticum</i>			R
			<i>Corynebacterium imitans</i>		R	
			<i>Corynebacterium jeikeium</i>		R	R
			<i>Corynebacterium mucifaciens</i>		R	
			<i>C. pseudogenitalium</i>			R
		Geodermatophilaceae	<i>Blastococcus saxobsidens</i>		R	
		Intrasporangiaceae	<i>Janibacter</i>		R	
		Microbacteriaceae	<i>Agrococcus jejuensis</i>	R		
			<i>Arthrobacter agilis</i>		R	
			<i>Microbacterium barkeri</i>		R	R
			<i>Microbacterium paraoxydans</i>		R	R
			<i>Zimmermannella bifida</i>		R	
		Micrococcaceae	<i>Micrococcus luteus</i>	R	R	R
		Mycobacteriaceae	<i>Mycobacterium flaterense</i>		R	
		Nocardiaceae	<i>Rhodococcus globerulus</i>	R	R	
		Propionibacteriaceae	<i>Microlunatus aurantiacus</i>			R
			<i>Propionibacterium acnes</i>	R	R	R
			<i>P. granulosum</i>		R	
		Pseudonocardiaceae	<i>Saccharopolyspora hirsuta</i>	R	R	R
		Streptomyetaceae	<i>Streptomyces thermovulgaris</i>	R		
		Williamsiaceae	<i>Williamsia muralis</i>			R
		Bifidobacteriaceae	<i>Bifidobacterium adolescentis</i>	R	R	R
			<i>Bifidobacterium bifidum</i>	R	R	R
			<i>Bifidobacterium breve</i>	R		R
			<i>Bifidobacterium longum</i>	R	R	R
			<i>B. pseudocatenulatum</i>	R	R	R
			<i>Bifidobacterium saeculare</i>	R		
			<i>Gardnerella vaginalis</i>			R
		Coriobacteriaceae	<i>Adlercreutzia equolifaciens</i>	R	R	R
			<i>Atopobium vaginae</i>		R	R
			<i>Collinsella aerofaciens</i>	R	R	C
			<i>Collinsella stercoris</i>		R	R
			<i>Eggerthella hongkongensis</i>	R		R
			<i>Eggerthella lenta</i>	R	R	R
			<i>Eggerthella sinensis</i>			R
			<i>Olsenella</i>			R
			<i>Slackia</i>	R		R
			<i>Bacteroides acidifaciens</i>		R	R
Bacteroidetes	Bacteroidia	Bacteroidaceae	<i>Bacteroides barnesi</i>			R
			<i>Bacteroides caccae</i>	R	R	R

<i>Bacteroides capillosus</i>	R	R	R
<i>Bacteroides cellulosilyticus</i>	R	R	R
<i>Bacteroides coprocola</i>	R	R	

Table S1 continued

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
			<i>Bacteroides coprophilus</i>		R	R
			<i>Bacteroides dorei</i>	R	C	R
			<i>Bacteroides eggerthii</i>	R	R	R
			<i>Bacteroides faecis</i>	R	R	R
			<i>Bacteroides fingoldii</i>		R	R
			<i>Bacteroides fragilis</i>	R	R	R
			<i>Bacteroides intestinalis</i>	R	R	R
			<i>Bacteroides massiliensis</i>	R	R	R
			<i>Bacteroides nordii</i>			R
			<i>Bacteroides ovatus</i>	R	R	R
			<i>Bacteroides plebeius</i>	R	R	R
			<i>Bacteroides salyersiae</i>	R	R	R
			<i>Bacteroides stercoris</i>	R	R	R
			<i>Bacteroides thetaiotaomicron</i>	R	R	R
			<i>Bacteroides uniformis</i>	R	C	C
			<i>Bacteroides vulgatus</i>	C	C	C
			<i>Bacteroides xylanisolvens</i>	R	R	R
		Porphyromonadaceae	<i>Dysgonomonas gadei</i>	R	R	C
			<i>Parabacteroides distasonis</i>	R	C	C
			<i>Parabacteroides goldsteinii</i>	R	R	R
			<i>Parabacteroides johnsonii</i>	R	R	R
			<i>Parabacteroides merdae</i>	R	R	C
			<i>Porphyromonas levii</i>	R	R	R
			<i>Porphyromonas somerae</i>	R	R	R
			<i>Porphyromonas uenonis</i>		R	R
			<i>Tannerella forsythia</i>	R	R	R
		Prevotellaceae	<i>Prevotella baroniae</i>	R	R	R
			<i>Prevotella bergensis</i>		R	R
			<i>Prevotella bivia</i>	R	R	R
			<i>Prevotella buccae</i>		R	
			<i>Prevotella buccalis</i>	R	R	R
			<i>Prevotella copri</i>	R	R	R
			<i>Prevotella corporis</i>	R	R	R
			<i>Prevotella dentasini</i>			R
			<i>Prevotella disiens</i>	R	R	R
			<i>Prevotella histicola</i>		R	R
			<i>Prevotella loescheii</i>			R
			<i>Prevotella melaninogenica</i>		R	R
			<i>Prevotella oulorum</i>		R	
			<i>Prevotella pallens</i>			R
			<i>Prevotella pleuritidis</i>		R	
			<i>Prevotella ruminicola</i>		R	R
			<i>Prevotella stercorea</i>	R	R	R
		Rikenellaceae	<i>Alistipes fingoldii</i>	R	R	R
			<i>Alistipes massiliensis</i>	R	R	R
			<i>Alistipes putredinis</i>	R	R	R
	Cytophagia	Cytophagaceae	<i>Hymenobacter</i>		R	R
		Flammeovirgaceae	<i>Rapidithrix thailandica</i>		R	

	Flavobacteriia	Flavobacteriaceae	<i>Chryseobacterium hominis</i>	R	R	
			<i>Chryseobacterium joostei</i>	R	R	
	Sphingobacteriia	Chitinophagaceae	<i>Chitinophaga arvensicola</i>	R		R
Deinococcus-Thermus	Deinococci	Deinococcaceae	<i>Deinococcus proteolyticus</i>	R		
Firmicutes	Bacilli	Alicyclobacillaceae	<i>Alicyclobacillus acidoterrestris</i>	R		
			<i>Alicyclobacillus vulcanalis</i>			R

Table S1 continued

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
		Bacillaceae	<i>Anoxybacillus kestanbolensis</i>		R	R
			<i>Bacillus cereus</i>	R	C	R
			<i>Bacillus fordii</i>		R	
			<i>Geobacillus stearothermophilus</i>		R	
		Paenibacillaceae	<i>Paenibacillus humicus</i>		R	
		Planococcaceae	<i>Viridibacillus arvi</i>		R	
		Staphylococcaceae	<i>Jeotgalicoccus halotolerans</i>		R	R
			<i>Macrococcus caseolyticus</i>	R		
			<i>Salinicoccus roseus</i>		R	
			<i>Staphylococcus aureus</i>	R		R
			<i>Staphylococcus epidermidis</i>	R	R	R
			<i>Staphylococcus hominis</i>	R		R
		Aerococcaceae	<i>Abiotrophia defectiva</i>	R	R	R
			<i>Aerococcus viridans</i>			R
			<i>Facklamia ignava</i>		R	
			<i>Facklamia languida</i>		R	
		Carnobacteriaceae	<i>Marinilactibacillus psychrotolerans</i>	R		R
		Enterococcaceae	<i>Enterococcus avium</i>		R	
			<i>Enterococcus cecorum</i>	R	R	R
			<i>Enterococcus durans</i>	R		
			<i>Enterococcus faecium</i>	R		R
			<i>Enterococcus lactis</i>	R		R
		Lactobacillaceae	<i>Lactobacillus alimentarius</i>		R	
			<i>Lactobacillus crispatus</i>	R		R
			<i>Lactobacillus delbrueckii</i>		R	
			<i>Lactobacillus gasseri</i>	R	R	R
			<i>Lactobacillus iners</i>	R		R
			<i>Lactobacillus intestinalis</i>	R	R	R
			<i>Lactobacillus manihotivorans</i>	R		
			<i>Lactobacillus mucosae</i>	R	R	R
			<i>Lactobacillus reuteri</i>		R	
			<i>Lactobacillus vaccिनosterus</i>	R		
			<i>Lactobacillus zeae</i>	R		
			<i>Pediococcus acidilactici</i>	R		
		Leuconostocaceae	<i>Weissella cibaria</i>	R	R	
			<i>Weissella confusa</i>	R		
		Streptococcaceae	<i>Lactococcus lactis</i>		R	R
			<i>Streptococcus anginosus</i>	R	R	R
			<i>Streptococcus bovis</i>	R		
			<i>Streptococcus dysgalactiae</i>			R
			<i>Streptococcus gallinaceus</i>			R
			<i>Streptococcus gordonii</i>			R
			<i>Streptococcus mitis</i>	R	R	R
			<i>Streptococcus oralis</i>	R	R	R
			<i>Streptococcus parasanguinis</i>	R	R	R

Clostridia	Clostridiaceae	<i>Streptococcus pluranimalium</i>			R
		<i>Streptococcus thermophilus</i>	R	R	R
		<i>Alkaliphilus transvaalensis</i>	R	R	R
		<i>Clostridium amygdalinum</i>	R	R	R
		<i>Clostridium asparagiforme</i>	R	R	R
		<i>Clostridium bartlettii</i>	R	R	R
		<i>Clostridium bolteae</i>	R	C	C
		<i>Clostridium butyricum</i>	R		
		<i>Clostridium celerecrescens</i>	R	R	R

Table S1 continued

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
			<i>Clostridium chauvoei</i>		R	R
			<i>Clostridium citroniae</i>	R	R	R
			<i>Clostridium cocleatum</i>	R	R	R
			<i>Clostridium difficile</i>	R		
			<i>Clostridium disporicum</i>	R		
			<i>Clostridium ghonii</i>	R		R
			<i>Clostridium hathewayi</i>	R	R	R
			<i>Clostridium hylemonae</i>			R
			<i>Clostridium indolis</i>	R	R	C
			<i>Clostridium innocuum</i>	R	R	R
			<i>Clostridium intestinale</i>			R
			<i>Clostridium lactatifermentans</i>	R	R	R
			<i>Clostridium lavalense</i>	R	R	R
			<i>Clostridium leptum</i>	R	R	R
			<i>C. methoxybenzovorans</i>	R	R	R
			<i>Clostridium methylpentosum</i>	R	R	C
			<i>Clostridium orbiscindens</i>	R	C	C
			<i>Clostridium paraputrificum</i>	R		
			<i>Clostridium perfringens</i>	R		
			<i>Clostridium puniceum</i>	R	R	R
			<i>Clostridium ramosum</i>	R	R	R
			<i>Clostridium saccharolyticum</i>	R	R	R
			<i>Clostridium scindens</i>	R		
			<i>Clostridium spiroforme</i>	R	R	R
			<i>Clostridium stercorearium</i>	R		
			<i>Clostridium straminisolvans</i>	R	R	R
			<i>Clostridium symbiosum</i>	R	C	R
			<i>Clostridium termitidis</i>		R	R
			<i>Clostridium thermocellum</i>	R	R	R
			<i>Clostridium xylanolyticum</i>	R	R	R
		Clostridiales Family XI	<i>Anaerococcus hydrogenalis</i>	R	R	R
			<i>Anaerococcus lactolyticus</i>			R
			<i>Anaerococcus octavius</i>	R	R	R
			<i>Anaerococcus prevotii</i>	R	R	R
			<i>Fingoldia magna</i>	R	R	R
			<i>Parvimonas micra</i>	R	R	R
			<i>Peptoniphilus asaccharolyticus</i>	R	R	R
			<i>Peptoniphilus ivorii</i>	R	R	R
			<i>Tissierella praeacuta</i>	R	R	R
			<i>Eubacterium infirmum</i>	R	R	R
		Clostridiales Family XIII	<i>Eubacterium minutum</i>		R	
			<i>Eubacterium sulci</i>	R	R	R

		<i>Mogibacterium pumilum</i>	R	R	R
Clostridiales Family XVIII		<i>Symbiobacterium thermophilum</i>		R	
Eubacteriaceae		<i>Eubacterium rectale</i>	C	C	C
		<i>Eubacterium siraeum</i>	R	R	R
Lachnospiraceae		<i>Anaerostipes caccae</i>		R	
		<i>Blautia producta</i>	R	C	C
		<i>Coprococcus catus</i>	R	R	R
		<i>Coprococcus comes</i>	R	R	R
		<i>Coprococcus eutactus</i>	R	R	C
		<i>Dorea formicigenerans</i>	R	R	R
		<i>Lachnospira pectinoschiza</i>	R	R	R
		<i>Moryella indoligenes</i>		R	R

Table S1 continued

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
			<i>Pseudobutyrvibrio fibrisolvens</i>	R	R	R
			<i>Pseudobutyrvibrio ruminis</i>	R	R	R
			<i>Roseburia faecis</i>	R	C	C
			<i>Roseburia hominis</i>	R	R	R
			<i>Roseburia intestinalis</i>	R	R	R
		Peptococcaceae	<i>Desulfitobacterium hafniense</i>		R	R
		Peptostreptococcaceae	<i>Eubacterium yurii</i>		R	R
			<i>Peptostreptococcus anaerobius</i>	R	R	R
			<i>Peptostreptococcus stomatis</i>		R	
		Ruminococcaceae	<i>Anaerotruncus colihominis</i>	R	R	R
			<i>Faecalibacterium prausnitzii</i>	C	C	C
			<i>Oscillospira guilliermondii</i>	R	C	C
			<i>Ruminococcus albus</i>	R	R	R
			<i>Ruminococcus bromii</i>	R	C	C
			<i>Ruminococcus callidus</i>	R	R	R
			<i>Ruminococcus flavefaciens</i>	R	R	R
			<i>Ruminococcus gnavus</i>	C	C	C
			<i>Ruminococcus obeum</i>	R	R	C
			<i>Ruminococcus torques</i>	R	R	C
		Syntrophomonadaceae	<i>Syntrophomonas curvata</i>			R
	Erysipelotrichi	Erysipelotrichaceae	<i>Catenibacterium mitsuokai</i>	R	R	R
			<i>Coprobacillus cateniformis</i>	R	R	R
			<i>Erysipelothrix rhusiopathiae</i>	R		
			<i>Eubacterium bifforme</i>	R	R	R
			<i>Eubacterium cylindroides</i>		R	R
			<i>Holdemania filiformis</i>	R	R	R
		Erythrobacteraceae	<i>Solobacterium moorei</i>		R	R
	Negativicutes	Acidaminococcaceae	<i>Acidaminococcus fermentans</i>	R	R	R
			<i>Acidaminococcus intestini</i>	R	R	R
			<i>Phascolarctobacterium</i>	R	R	R
		Veillonellaceae	<i>Dialister invisus</i>	R	R	C
			<i>Dialister micraerophilus</i>	R	R	R
			<i>Dialister pneumosintes</i>	R	R	
			<i>Dialister succinatiphilus</i>	R	R	R
			<i>Megamonas hypermegale</i>		R	R
			<i>Megasphaera elsdenii</i>	R	R	R
			<i>Mitsuokella jalaludinii</i>		R	R
			<i>Mitsuokella multacida</i>	R		R
			<i>Sporomusa aerivorans</i>	R		

Fusobacteria	Fusobacteria	Fusobacteriaceae	<i>Veillonella dispar</i>	R	R	R
			<i>Veillonella parvula</i>	R	R	R
			<i>Victivallis vadensis</i>		R	R
			<i>Fusobacterium gonidiaformans</i>		R	R
			<i>Fusobacterium nucleatum</i>		R	
			<i>Fusobacterium periodonticum</i>	R	R	R
			<i>Fusobacterium ulcerans</i>	R	R	R
		Leptotrichiaceae	<i>Leptotrichia goodfellowii</i>	R		
			<i>Sneathia amnionii</i>	R	R	
			<i>Sneathia sanguinegens</i>			R
Planctomycetes	Planctomycetia	Planctomycetaceae	<i>Gemmata obscuriglobus</i>	R		
Proteobacteria	Alphaproteobacteria	Caulobacteraceae	<i>Asticcacaulis excentricus</i>		R	
			<i>Brevundimonas nasdae</i>			R
		Bradyrhizobiaceae	<i>Nitrobacter vulgaris</i>			R
			<i>Rhodopseudomonas</i>	R	R	R

Table S1 continued

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
		Methylobacteriaceae	<i>Methylobacterium tardum</i>	R	R	
		Methylocystaceae	<i>Methylocystis parvus</i>			R
		Phyllobacteriaceae	<i>Mesorhizobium loti</i>			R
			<i>Phyllobacterium myrsinacearum</i>	R	R	R
		Rhodobacteraceae	<i>Labrenzia aggregata</i>		R	
			<i>Loktanella marincola</i>		R	
			<i>Paracoccus alcaliphilus</i>	R	R	
			<i>Paracoccus koreensis</i>	R	R	
			<i>Paracoccus marcusii</i>	R		R
			<i>Paracoccus yeei</i>	R	R	R
			<i>Rubellimicrobium</i>	R	R	
			<i>Rhodocista pekingensis</i>			R
			<i>Neorickettsia risticii</i>	R	R	R
		Erythrobacteraceae	<i>Porphyrobacter tepidarius</i>		R	
		Sphingomonadaceae	<i>Sphingobium yanoikuyae</i>			R
			<i>Sphingomonas asaccharolytica</i>		R	R
			<i>Sphingomonas faeni</i>			R
	Betaproteobacteria	Comamonadaceae	<i>Acidovorax facilis</i>	R	R	R
		Oxalobacteraceae	<i>Massilia timonae</i>	R	R	R
		Sutterellaceae	<i>Sutterella parvirubra</i>	R	R	R
			<i>Sutterella wadsworthensis</i>	R	C	C
		Burkholderiaceae	<i>Pandoraea pulmonicola</i>			R
			<i>Ralstonia pickettii</i>	R	R	R
		Comamonadaceae	<i>Hydrogenophaga taeniospiralis</i>		R	
			<i>Variovorax paradoxus</i>	R		
		Hydrogenophilaceae	<i>Hydrogenophilus</i>			R
		Neisseriaceae	<i>Microvirgula aerodenitrificans</i>		R	
			<i>Neisseria mucosa</i>			R
			<i>Neisseria subflava</i>			R
		Burkholderiaceae	<i>Cupriavidus taiwanensis</i>	R	R	
		Comamonadaceae	<i>Comamonas denitrificans</i>	R	R	
			<i>Comamonas terrigena</i>		R	
			<i>Comamonas testosteroni</i>	R	R	
			<i>Dechloromonas</i>		R	
	Deltaproteobacteria	Desulfovibrionaceae	<i>Bilophila wadsworthia</i>		R	R
			<i>Desulfovibrio aespoeensis</i>	R		

			<i>Desulfovibrio piger</i>	R	R	R
	Myxococcaceae		<i>Anaeromyxobacter dehalogenans</i>	R		
Epsilonproteobacteria	Campylobacteraceae		<i>Campylobacter concisus</i>	R		
			<i>Campylobacter faecalis</i>			R
			<i>Campylobacter hominis</i>	R	R	R
			<i>Campylobacter mucosalis</i>		R	
			<i>Campylobacter ureolyticus</i>	R	R	R
Gamma	Aeromonadaceae		<i>Aeromonas media</i>	R	R	R
proteobacteria	Succinivibrionaceae		<i>Succinivibrio</i>			R
	Shewanellaceae		<i>Shewanella putrefaciens</i>		R	R
	Enterobacteriaceae		<i>Shigella boydii</i>	R	R	R
			<i>Shigella dysenteriae</i>	R	R	R
			<i>Shigella flexneri</i>	C	C	C
			<i>Shigella sonnei</i>	R	R	R
			<i>Citrobacter freundii</i>	R	R	R
			<i>Cronobacter muytjensii</i>	R	R	R
			<i>Cronobacter turicensis</i>	R		
			<i>Enterobacter hormaechei</i>	R	R	R

Table S1 continued

Phylum	Class	Family	Taxon Name	Crohn's	Siblings	Healthy
			<i>Escherichia coli</i>	C	R	R
			<i>Escherichia fergusonii</i>	C	C	C
			<i>Klebsiella granulomatis</i>	R	R	R
			<i>Klebsiella pneumoniae</i>	R	R	R
			<i>Klebsiella variicola</i>	R	R	R
			<i>Kluyvera ascorbata</i>	R		
			<i>Morganella morganii</i>	R		
			<i>Raoultella planticola</i>	R		R
		Oceanospirillaceae	<i>Marinospirillum insulare</i>		R	
		Pasteurellaceae	<i>Actinobacillus porcitonisillarum</i>			R
			<i>Haemophilus parainfluenzae</i>	R	R	R
		Moraxellaceae	<i>Acinetobacter johnsonii</i>	R	R	R
			<i>Acinetobacter junii</i>	R	R	R
			<i>Acinetobacter lwoffii</i>	R		R
			<i>Acinetobacter radioresistens</i>	R		
			<i>Acinetobacter schindleri</i>		R	
			<i>Enhydrobacter aerosaccus</i>		R	R
		Pseudomonadaceae	<i>Pseudomonas aeruginosa</i>	R	R	R
			<i>Pseudomonas geniculata</i>	R	R	C
			<i>Pseudomonas mendocina</i>		R	
			<i>Pseudomonas putida</i>	R	R	R
			<i>Pseudomonas veronii</i>		R	R
		Xanthomonadaceae	<i>Stenotrophomonas</i>		R	R
			<i>Thermomonas dokdonensis</i>			R
			<i>Xanthomonas vesicatoria</i>	R	R	R
Spirochaetes	Spirochaetes	Brachyspiraceae	<i>Brachyspira aalborgi</i>			R
Synergistetes	Synergistia	Synergistaceae	<i>Jonquetella anthropi</i>		R	
			<i>Pyramidobacter piscolens</i>		R	R
			<i>Synergistes jonesii</i>			R
Tenericutes	Mollicutes	Acholeplasmataceae	<i>Acholeplasma</i>		R	
			<i>Acholeplasma parvum</i>		R	R
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiaceae	<i>Akkermansia muciniphila</i>	R	R	R

569 **Supplementary methods**

570 Peripheral blood T-cell flow cytometry

571 The fluorescently labeled antibodies used were: anti-CD3 Pacific Blue (clone OKT3, Biolegend, San Diego,
572 CA,USA), anti-CD45RA PE-Cy7 (clone L48, BD Bioscience, Franklin Lakes, NJ, USA), anti-CD8 PerCP-Cy5.5
573 (clone SK1, BD Bioscience) and anti-CD4 APC (clone RPA-T4, BD Bioscience), anti- β 7 PE (clone FIB504, BD
574 Pharmingen).

575 Isotype-matched controls for mIgG1k PE-Cy7 (clone MOPC-21, BD Pharmingen), rat IgG2a PE (clone R35-95,
576 BD Bioscience), mIgG1 PE (clone MOPC-21, BD Bioscience), rIgM FITC (clone R4-22, BD Pharmingen) and
577 mIgG1 FITC (clone MOPC-21, BD Pharmingen) were used to set positive and negative regions for gating
578 during analysis. Anti-CD8 FITC (clone LT8, AbD Serotec, Kidlington, UK), anti- β 7 PE (clone FIB504, BD
579 Pharmingen), anti-CD8 PerCP-Cy5.5 (clone SK1, BD Bioscience), anti-CD45RA PE-Cy7 (clone L48, BD
580 Bioscience) anti-CD3 PB (clone OKT3, Biologend) and anti-CD4 APC (clone RPA-T4, BD Bioscience)
581 conjugated antibodies were used for off-line compensation.

582

583 Gut mucosal microbiota

584 **DNA extraction protocol**

585 Biopsy DNA extraction was carried out using a phenol/chloroform based method, as follows: Guanidinium
586 thiocyanate–EDTA–sarkosyl (500 μ L) and PBS (500 μ L), pH 8.0, were added to biopsy samples. Cell
587 disruption was achieved using a Fastprep-24 Instrument (MP Biomedicals Europe, Illkirch, France) 6.5 m/s,
588 60 s, followed by incubation at 90 °C for 1 min and –20 °C for 5 min. Cell debris was pelleted by
589 centrifugation at 12 000 \times g for 2 min at 4 °C. Supernatant was transferred to a fresh microfuge tube. NaCl
590 (to a final concentration of 0.5 mol/L and polyethylene glycol (to a final concentration of 15%) were added
591 and DNA precipitated at 4 °C for 30 min. DNA was pelleted by centrifugation at 12 000 \times g for 2min at 4 °C
592 and resuspended in 300 μ L of sterile distilled water. Samples were heated at 90 °C for 30 s and vortexed.

593 Phenol/chloroform (1:1) (300 µL) was added, and samples were vortexed for 20 s before centrifugation at
594 12 000 × g at 4 °C for 3min. The upper phase was then transferred to a fresh microfuge tube. Total DNA was
595 then precipitated by the addition of an equal volume of isopropanol, a 0.1-volume 10 mol/L ammonium
596 acetate, and 1 µL of GenElute linear polyacrylamide (Sigma-Aldrich, Gillingham, UK) and incubated at –20 °C
597 for 25 min. DNA was pelleted by centrifugation at 12 000 × g at 4 °C for 5 min. Pelleted DNA was then
598 washed 3 times in 70% ethanol, dried, and resuspended in 50 µL of sterile distilled water. DNA extracts
599 were quantified using the Picodrop Microlitre Spectrophotometer (GRI, Braintree, UK). Negative controls,
600 consisting of sterile water, were included in the PMA treatment, DNA extraction, and PCR amplification
601 steps.

602 16S rRNA gene sequencing

603 Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed as described previously using
604 Gray28F 5'-TTTGATCNTGGCTCAG-3' and Gray519r 5'-GTNTTACNGCGGCKGCTG-3').¹ A single-step 30 cycle
605 PCR using HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA) performed under the following conditions:
606 94°C for 5minutes, followed by 28 cycles of: 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1
607 minute. Amplification was followed by a final elongation step at 72°C for 5 minutes. Following PCR, all
608 amplicon products from different samples were mixed in equal concentrations and purified using
609 Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced utilizing
610 Roche 454 FLX titanium instruments and reagents following manufacturer's guidelines.

611 Sequence data analysis was carried out. Here, the Q25 sequence data derived from the sequencing process
612 was processed using standard analysis pipeline processes (MR DNA, Shallowater, USA). Sequences were
613 depleted of barcodes and primers then short sequences, 200 bp removed, as were sequences with
614 ambiguous base calls removed, and sequences with homopolymer runs exceeding 6 bp, sequences were
615 denoised and chimeras removed.²⁻⁸ Operational taxonomic units were defined after removal of singleton
616 sequences, clustering at 3% divergence (97% similarity). Final OTUs were taxonomically classified using
617 BLASTn against a curated databased derived fromGreenGenes, NCBI and RDP databases.⁹ Normalized and

618 de-noised files were then rarefied and run through QIIME¹⁰ to generate alpha and beta diversity data.
619 Additional statistical analyses were performed with NCSS2007 (NCSS, UT) and XLstat 2012 (Addinsoft, NY).

620 **References for supplementary methods**

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